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The vagus nerve mediates cardio-respiratory coupling that changes with metabolic demand in a temperate notothenioid fish

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Summary

The extent and efficiency of cardio-respiratory coupling (CRC) in teleost fishes is unclear. We simultaneously monitored heart rate (f_H) and ventilation rate (f_V) in *Paranotothenia angustata,* and applied modern power spectral analysis (PSA) mathematics to examine the rate association under varying levels of oxygen consumption ($\dot{M}_{O_2}$). At low $\dot{M}_{O_2}$ (0.94 mmol O_2 kg^{-1} h^{-1}) there was a correspondingly low f_H and f_V (25.5±2.4 min^{-1} and 29.2±2.6 min^{-1}, respectively). Heart rate variability (HRV) consisted of oscillatory components caused by periodic vagal inhibition of the heart beat. Cross-spectral analysis showed that f_H and f_V were coupled, with the response lag in heart beat being approximately one seventh of each ventilation cycle. Ingestion of food elevated $\dot{M}_{O_2}$ (1.99±0.02 mmol O_2 kg^{-1} h^{-1}) and increased both f_H and f_V (45±2.3 min^{-1} and 52±2 min^{-1}, respectively, $P<0.05$), but CRC was maintained despite a reduction in HRV. The elevated stress caused by handling and placement of fish into respirometry chambers raised f_H and f_V to a similar rate as observed after feeding, although high-frequency (>0.2 Hz) oscillations in f_H were lacking and $\dot{M}_{O_2}$ was lower (1.82±0.03 mmol O_2 kg^{-1} h^{-1}, $P<0.05$). Subsequent cardiac vagotomy elevated f_H and f_V (55.5±0.8 min^{-1} and 48.2±0.7 min^{-1}, respectively; $P<0.05$) but abolished all HRV and CRC, although $\dot{M}_{O_2}$ was significantly less for a given f_H and f_V compared to intact fish. Thus, *P. angustata* exhibits vagally mediated CRC, and the association between f_H and f_V varies according to oxygen demand.

Key words: power spectral analysis, heart rate variability, ventilation rate variability, ECG.

Introduction

To satisfy metabolic demand animals have evolved efficient systems for oxygen uptake from the surrounding environment by diffusion *via* lungs or gills, and delivery by convection *via* the circulatory system. In mammals, there is coordination between the cardiac and ventilation cycles, a phenomenon known as respiratory sinus arrhythmia (RSA). This is mediated by the Xth cranial (vagus) nerve, resulting in tachycardia on inspiration and bradycardia on expiration, and a matching of air and blood flow at the lungs to optimise oxygen extraction (Dejours, 1988; Taylor et al., 1999). Water contains significantly less oxygen per unit volume than air and is considerably more viscous and dense. Consequently, fish require a relatively greater energy expenditure to extract sufficient oxygen to fuel aerobic metabolism, compared with air breathers (Dejours, 1988). Therefore, similar physiological mechanisms may exist if selection pressure acted to minimise cost/maximise the effectiveness of oxygen transfer from water to blood (Hughes and Shelton, 1962; Piper and Scheid, 1982). Whether a similar central drive for cardio-respiratory coupling (CRC) operates in fish is still not clear, though recent data suggests that an analogous drive may indeed exist (Campbell et al., 2005a).

Modulation of cardiac output and systemic or branchial vascular resistance by both parasympathetic and sympathetic fibres has been recorded in a number of fish species (Nilsson, 1983; Morris and Nilsson, 1994; Farrell, 1991). Furthermore, efferent activity from the cardiac vagus is synchronised with the buccal opening phase of the ventilation cycle in tench (Randall, 1966), and cholinergic vasomotor fibers in the vagus innervate sphincters at the base of efferent filament arteries that regulate perfusion of the branchial circulation (Nilsson, 1983). Moreover, a 1:1 synchronous relationship between heart rate (f_H) and ventilation (f_V) occurred in Atlantic salmon under moderate hypoxia, and the muscarinic receptor antagonist atropine abolished this synchrony, suggesting a fundamental role for the vagus in controlling CRC (Randall and Smith, 1967; Smith and Davie, 1984).

Mathematical models have suggested, however, that there is no need for CRC in aquatic breathers, because pulsatile synchrony of blood and water flow would provide a marginal advantage to gas exchange efficiency (Malte, 1992). Furthermore, whilst the frequency of respiratory driven oscillations in f_H (RSA) can be identified in mammals using power spectral analysis (PSA), the same has not been shown for fishes (DeVera and Priede, 1991; Altimiras et al., 1995;

Campbell et al., 2004). Power spectra generated from mammalian f_H traces show a fundamental component at the frequency of ventilation due to a periodic slowing of f_H (Malik, 1996). In contrast, spectra derived from f_H traces from fish are characterised by multiple peaks at frequencies well below that of f_V. It was thus concluded that there is no evidence for centrally generated, respiration related periodicity in f_H (DeVera and Priede, 1991; Altimiras et al., 1995). However, recent developments in the application of spectral statistics to f_H signals from fish have found confounding factors in the original analytical technique due to a process known as aliasing, which may lead to erroneous data interpretation (Campbell et al., 2006; Taylor et al., 2006). Aliasing occurs when an oscillatory component is identified at a lower frequency than its true frequency, and will occur whenever $f_V > f_H/2$. This is a typical situation within fishes, and the mathematical complications this imposes on analysis has already been explored (for details, see Campbell et al., 2006). An alternative technique was proposed where the Fourier transform is applied to the raw electrocardiogram (ECG) signal instead of the usual interbeat (R-R interval) tachogram, and this revealed the existence of CRC in resting fish that may be analogous to the RSA of mammals (Campbell et al., 2005a). The present study utilised this new PSA approach to test the hypothesis that cardio-respiratory coupling is modified in fish according to metabolic demand.

Paranotothenia angustata is a suitable test species based on a number of criteria: (1) it has ventilatory oscillations in the f_H signal that can be identified using the refined PSA algorithm (Campbell et al., 2006); (2) the notothenioid group of fishes rely to a large extent on frequency modulation of cardiac output (Axelsson et al., 1992) (H.A.C. and S.E., unpublished data) and therefore changes in f_H, as opposed to stroke volume, are likely to be associated with altering blood flow to the gills; (3) the fish will remain motionless on the bottom of a respirometry chamber and therefore enable uninterrupted measurement of heart beat sequences, a requirement for robust power spectral analysis. Cardio-respiratory parameters were recorded simultaneously using implanted electrodes, and concurrent recording of $\dot{M}_{O_2}$ made by static closed circuit respirometry. It is well documented that after handling and placement in a respirometer, fish show an elevated $\dot{M}_{O_2}$ that gradually reduces to a resting rate over succeeding days (Steffensen et al., 1994). This metabolic response was exploited to measure f_H and f_V as $\dot{M}_{O_2}$ varied, and compared to an elevated metabolic demand induced by feeding. The animals also underwent surgical ablation of the cardiac branch of the vagus nerve to examine how $\dot{M}_{O_2}$ is associated with the induced changes in cardio-respiratory parameters.

Materials and methods

Animals, surgery and protocol

Paranotothenia angustata Hutton 1875 (N=12) were caught by baited pot outside the mouth of Otago harbour, New Zealand. They were transport back to Portobello Marine Laboratory and acclimated for 7 days in a 3000 l outside pond that was flushed with aerated, filtered seawater at ambient temperature (10±0.8°C, mean ± s.e.m.). Fish were carefully removed from the holding facility, anaesthetised in MS222 (0.3 mg l^{-1} seawater), and placed dorsal side up on an operating table and the gills irrigated with a less concentrated MS222 (0.1 mg l^{-1}) solution. Recording electrodes (7-strand Teflon coated wire, length 40 cm, diameter 0.2 mm; A-M Systems, Carlsborg, WA, USA) were hooked into the end of a 24 G hypodermic needle and inserted through the opercular septum at the base of the left fourth gill arch. The wires were placed 2 cm apart and advanced 2 mm through the septum, and care was taken to ensure that the pericardial membrane was not pierced. The trailing wire was attached by a single suture to the flank of the fish just dorsal of the opercular flap. Fish were recovered in aerated seawater and whilst still docile placed into a respirometery chamber. ECG wires exited out of a chimney in the top of the chamber.

The experimental protocol consisted of three phases for each fish. The first phase monitored heart rate (f_H), ventilation rate (f_V) and oxygen consumption ($\dot{M}_{O_2}$) for 120 h after anaesthesia, surgery and placement of fish in the respirometer. The second phase measured the same parameters for a further 120 h after each fish had ingested a 5% body mass (b.m.) ration (delivered through a chimney in the respirometry chamber). The third phase required the fish to be removed from the chamber, anaesthetised and undergo surgery to bilaterally section the vagal nerve, thereafter f_H and f_V as $\dot{M}_{O_2}$ were measured for 120 h post-surgery.

Recording of f_H and f_V

Bipolar ECG signals were recorded using a bioamplifier interfaced with a digital recording system (PowerLab, AD Instruments, Oxford, UK), sampling at 400 Hz. ECG and opercular movements could be observed as different frequency components transposed onto a single trace. That apparent ventilation signals were true opercular movements was confirmed by pilot experiments in a fish that was also fitted with a cannula to monitor opercular pressure. Both the timing of each heart beat and the occurrence of each ventilation cycle could be extracted from a single trace by the use of digital filtering techniques (Chart 5, AD Instruments, Oxford, UK). Uninterrupted recordings were made from each fish for 30 min twice every 24 h.

Measurement of oxygen consumption

The respirometry chamber was constructed from PVC pipe (8 cm×32 cm diameter×length) with O-ring sealed threaded end caps and had a final volume of 5 l. A chimney (3 cm×5 cm diameter×length) on the top of the chamber acted as an outflow during chamber flushing and allowed for the exit of ECG electrodes. Four chambers contained fish and a fifth was left blank to allow subtraction of bacterial $\dot{M}_{O_2}$. All chambers were immersed into an aerated water bath (10±0.4°C) that was continually flushed with aerated filtered seawater. Each chamber was fitted with two submersible pumps (100 l h^{-1}, Interpet, Birmingham, UK); one circulated the water around the chamber whilst the other flushed the chamber with aerated water from the water bath. During chamber oxygen measurements the flush pump was switched off. The water was extracted automatically from the chamber *via* a rotor valve (Omnifit, Birmingham, UK) and injected into a purpose-made

1 cm flow cell containing a 1508 bipolar oxygen electrode (Strathkelvin, Glasgow, UK). Automated recordings of the partial pressure of oxygen were made. The P_{O_2} was measured within each chamber once a second for a 10 min period. Calculation of fish oxygen consumption ($\dot{M}_{O_2}$) was made from the change in partial pressure of dissolved oxygen in the seawater at the experimental temperature, correcting for the fish body mass and the volume of water in the chamber taking into account water displacement. Further correction was made for differences in fish size using the recommended body mass exponent of 0.8, for demersal fish (Johnston and Battram, 1993).

Sectioning of the vagal nerve

For the third phase of study the fish were removed from the respirometry chamber and anaesthetised (MS222, 0.3 mg l^{-1}), before undergoing surgery to bilaterally section the cardiac branch of the vagal nerve trunk (cranial nerve X). This branch was identified in pilot experiments using electrical stimulation to stop the heart when identified (Campbell et al., 2004). After a 2 cm incision was made in the integument behind the fourth gill arch, the cardiac vagus could be seen branching from the pennate vagal trunk, and was sectioned by removing a 0.2 cm length. After suturing, fish were returned to the respirometer for recordings of ECG, ventilation and oxygen consumption.

Measurements were taken from 12 fish, but only eight were used for the final analysis as two individuals did not accept food in the respirometer, and two underwent surgery where the vagal nerve was exposed but not sectioned (sham operated controls). After the study fish were killed by MS222 overdose and whole body mass recorded (699±5 g; mean ± s.e.m., N=12). All experiments were conducted in accordance with the UK Animals (Scientific Procedures) Act of 1986.

Data analysis

To determine whether each heart beat was dependant on the preceding or succeeding ventilation cycle, it was necessary to study fluctuations in the respective periodicities. Therefore, results for heart and ventilation rates are expressed as the time interval between one heart beat or opercular movement and the next. This is termed the R-R interval and V-V interval, respectively. To provide a statistically testable unit of variability, data was first transformed using the non-linear mathematical techniques described below. The results from each non-linear transformation are shown graphically. It was only after transformation that individual fish data were grouped to produce testable means; these are displayed in the tables. To identify significant factors among mean cohorts, multi-factoral ANOVA with the F-test was used, and Fisher's least-significance difference procedure was used to discriminate amongst means (P<0.05).

Variability analysis

The within individual variability between heart beats and ventilation cycles was assessed for the degree of long-term or short-term variation using the following equations (Brennan et al., 2001): (1) standard deviation of the R-R (or V-V) interval (SDRR or SDVV), defined as the square root of the variance in intervals, e.g. where the mean R-R interval is denoted by E(R-R_n):

$$\text{SDRR} = \sqrt{E(\text{R-R}_n^2) - \text{R-R}^2}\ ; \quad (1)$$

(2) standard deviation of the successive differences of the R-R (or V-V) intervals (SDSD). It is defined as the square root of the variance of the sequence (statistical equivalent of root-mean square):

$$\text{SDSD} = \sqrt{\text{E(R-R}_n^2) - \text{R-R}_n^2}\ . \quad (2)$$

To determine the importance of the short-term variability in influencing oxygen consumption, it was expressed as the ratio (SDRR:SDSD). Significant differences between treatments were then assessed using multi-factoral ANOVA (P<0.05).

Power spectral analysis

Power spectral analysis (PSA) was applied to assess if the variability in the heart rate and ventilation rate was oscillatory in nature. The analytical procedures of frequency domain analysis for f_H are used routinely in clinical medicine, and a number of reviews discuss the theoretical basis of this (Baselli et al., 1986; Baselli et al., 1988; Malik, 1996). Statistical methods used in this study were adapted for cardiac signals from fish, and detailed methods have been presented elsewhere (Campbell et al., 2006). Briefly, the method takes a section of 256 consecutive R-R or V-V intervals, without artifacts or ectopic beats, and the Fourier transform (FT) is applied to the raw ECG or ventilation waveform. This technique has the advantage of a much higher sampling rate (500 Hz), compared to the usual method where the FT is applied to an R-R tachogram, in which case sampling rate is set by f_H. The Fourier transform was applied with a Hanning window to reduce spectral leakage, and the output spectra displayed graphically.

To test for statistical significant between treatments each spectrum was divided in exactly half and the power of the lower frequency half of the spectrum (LF) and the higher frequency half (HF) determined. The LF:HF ratio was then calculated, and provided a measure of the dominance of oscillatory components in both heart rate and ventilation (Campbell et al., 2005a). The mean was calculated for each treatment, and differences determined by multi-factor ANOVA.

Cross spectral analysis

To determine whether observed oscillations in heart beat or ventilation shared any temporal association, cross-spectral analysis was undertaken. This technique applies the FT to the raw ECG and ventilation cycle data and compares the amplitude and frequency association between spectral components. The coherence shows the extent of linearity between f_H and f_V at each frequency, giving values between 0 (i.e. no relationship) and 1 (i.e. maximal coherence). The phase relationship between f_H and f_V spectral components has a range between –180° and +180° (i.e. 0° will indicate that the oscillations are occurring simultaneously, and 180° completely out of phase). This will show the lead or lag of one component in f_H with respect to that of a similar component with high coherence in f_V.

To test for statistical significance between treatments the coherence and phase data was divided into low, mid and high

frequency bins. These were then grouped into cohorts of physiological state, and mean differences determined using multi-factoral ANOVA (P<0.05).

Results

Rate analysis

At rest, *P. angustata* has a low $\dot{M}_{O_2}$ (0.94 mmol O_2 kg^{-1} h^{-1}) with a correspondingly low f_H and f_V (25.5±2.4 min^{-1} and 29.2±2.6 min^{-1}, respectively). Handling and feeding both caused an elevation in $\dot{M}_{O_2}$, f_H and f_V. After initial placement in the respirometer the mean $\dot{M}_{O_2}$ was 1.82±0.03 mmol O_2 kg^{-1} h^{-1}, decreasing to 0.91±0.1 mmol O_2 kg^{-1} h^{-1} (± s.e.m., N=8; P<0.5) after 120 h (circles, Fig. 1). After feeding a 5%b.m. ration $\dot{M}_{O_2}$ reached a peak response between 4–6 h (1.99±0.02 mmol O_2 kg^{-1} h^{-1}), which was significantly (t=5.04, P<0.05) higher than previously shown by the same fish when first placed in the respirometer (squares, Fig. 1). $\dot{M}_{O_2}$ reached pre-feeding rates after ~72–96 h. During periods of elevated $\dot{M}_{O_2}$, f_H was higher than f_V, but as $\dot{M}_{O_2}$ declined f_H decreased (slope=3.6 and 3.3 for stressed and fed fish, respectively) more quickly than did f_V (slope=2.8 for both groups). Therefore, at a low $\dot{M}_{O_2}$, f_V was greater than f_H.

Sectioning the cardiac vagus and then replacing the fish into the respirometer produced an $\dot{M}_{O_2}$ that was significantly (t=8.7, P<0.05) less than that observed when the fish were first placed in the respirometer with the vagus intact (1.45± 0.025 mmol O_2 kg^{-1} h^{-1}). However, f_H was significantly higher than observed in intact fish, while f_V was unchanged. 120 h after surgery, both f_H and f_V had only decreased slightly, and therefore were ~twofold greater than that observed in an intact unfed fish after 120 h in the respirometer (triangles, Fig. 1).

The sham-operated control fish (vagus exposed but not sectioned) showed a comparable R-R and V-V interval with that of intact disturbed fish, and an $\dot{M}_{O_2}$ of 1.86± 0.08 mmol O_2 kg^{-1} h^{-1} (mean ± s.e.m., N=2), consistent with previous findings that surgical intervention has no discernable effect on recovered fish (Campbell et al., 2004).

Nonlinear geometric analysis

To provide a visual representation of the f_H and f_V variability, each R-R and V-V interval was plotted against the succeeding interval as a scattergram (Fig. 2). The four panels provide a quantitative display of how heart beat and ventilation cycle variability patterns change at different levels of oxygen consumption. Data from a single fish are shown for illustrative purposes, with mean long-term and short-term variability derived from Eqn 1 and Eqn 2 shown in Table 1.

Recordings from fish with high $\dot{M}_{O_2}$ after handling showed a narrowing in the lengthwise spread of R-R interval data along the diagonal axis from the origin (OA, Fig. 2A), illustrating that long-term (lt) HRV was greater than short-term (st) beat-to-beat changes. The mean group data showed a similar result, and the lt-HRV:st-HRV was >1 (Table 1). A similar pattern was observed for the V-V interval data (Fig. 2A). After 120 h undisturbed rest the plot of R-R intervals had spread along the axis normal to the diagonal, across the maximum cluster width (MA, Fig. 2B), indicating increased beat-to-beat short-term variability. The mean lt-HRV:st-HRV ratio had reversed to now be <1 (P<0.05, Table 1). The variability between V-V intervals was similar to the f_H, and mean lt-VRV:st-VRV was <1 (P<0.05, Table 1). Feeding caused a reduction in the spread of the R-R interval data along both the maximum and diagonal axes (Fig. 2C). However, the mean lt-HRV:st-HRV remained <1 (P<0.05, Table 1), indicating that beat-to-beat (short term) variability still dominated overall HRV. There was no change

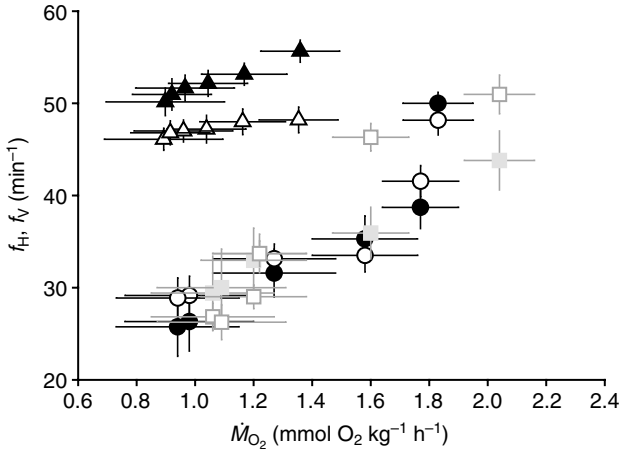

Fig. 1. Changes in $\dot{M}_{O_2}$ and associated f_H (filled symbols) and f_V (open symbols) measured simultaneously for 10 min three times daily in 12 *N. angustata* (mean ± s.e.m., n=12, N=36 observations), subjected to the following sequential measurements. Circles, recordings made for 120 h following initial anesthesia, surgery and placement in the respirometry chamber; squares, recordings taken for the subsequent 120 h after ingestion of a 5%b.m. food ration; triangles, daily recordings made in the same fish after it had returned to pre-fed values and had then undergone anaesthesia, bilateral section of the vagal nerve and placement back in the respirometry chamber.

Table 1. *Long-term and short-term variability in heart rate (HRV) and ventilation rate (VRV) calculated from fish with different rates of oxygen consumption*

Group	lt-HRV (ms)	st-HRV (ms)	lt-HRV:st-HRV
f_H			
A	59.5±3.4	52.1±4.1	1.14±0.2
B	333.2±12.2	447.3±28.3	0.74±0.2*
C	169.0±7.2	190.0±9.2	0.88±0.2*
D	13.8±0.4	12.8±0.7	1.08±0.1
f_V	lt-VRV (ms)	st-VRV (ms)	lt-VRV:st-VRV
A	57.2±4.2	56.7±3.6	1.00±0.08*
B	85.6±5.2*	97.1±4.3*	0.88±0.10*
C	81.8±3.4*	94.9±4.2*	0.87±0.10*
D	32.1±1.2	33.0±1.2	0.97±0.07*

f_H, heart rate; f_V, ventilation rate (means ± s.e.m., N=8) from fish with (A) a high $\dot{M}_{O_2}$ due to surgery and placement in a respirometer; (B) a low $\dot{M}_{O_2}$ after 120 h undisturbed starvation; (C) a high $\dot{M}_{O_2}$ due to ingestion of a 5%b.m. ration; (D) a high $\dot{M}_{O_2}$ due to bilateral vagotomy.

Heart beat interval (R-R) and ventilation cycle (V-V) variability was calculated using Eqn 1 and Eqn 2. Multivariate ANOVA assumes that the mean data for each physiological state is significantly different (P<0.05), and homogeneous groups are indicated by asterisks. lt, long-term; st, short-term.

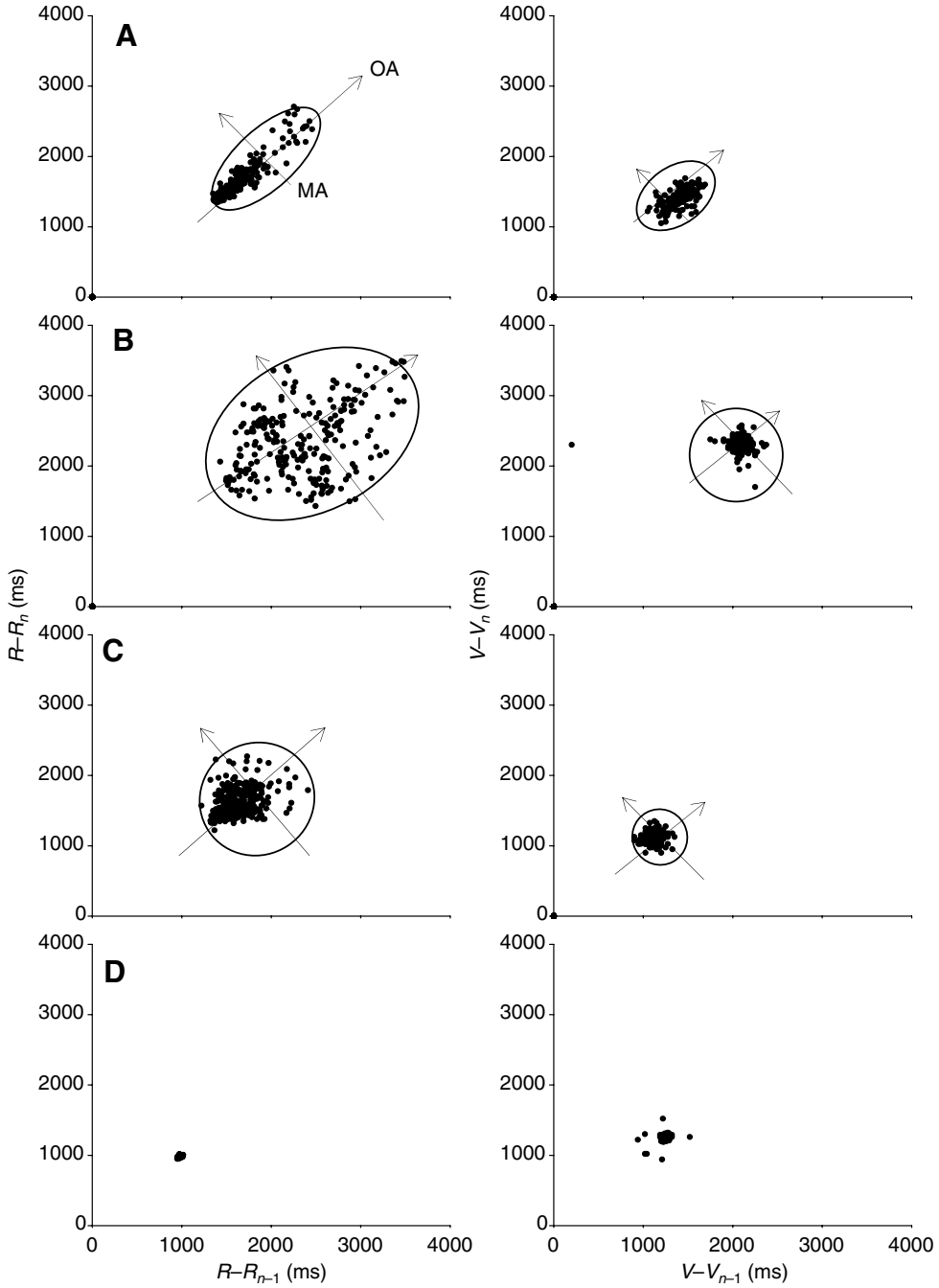

Fig. 2. Scattergram plots of 256 consecutive heart beat intervals (R-R) and ventilation cycles (V-V) plotted against its preceding interval (n–1). All plots are from a single individual: (A) with a high $\dot{M}_{O_2}$ due to surgery and placement in the respirometer; (B) a low $\dot{M}_{O_2}$ after 120 h undisturbed starvation; (C) a high $\dot{M}_{O_2}$ due to ingestion of a 5%b.m. ration; (D) a high $\dot{M}_{O_2}$ due to bilateral vagotomy. Each graph is fitted with an ellipse and axis orientated with the line of identity. Long-term heart rate variability runs along the diagonal axis from the origin (OA), and beat-to-beat variations occur along the normal to the axis maxima (MA).

in variability patterns of the V-V interval (lt-VRV:st-VRV <1; P<0.05, Table 1).

Bilateral vagotomy significantly reduced both short- and long-term variability in both f_H and f_V (Fig. 2D). In contrast to intact animals, the short-term variability signal from the f_V was greater than the variability in f_H (SDVV:SDSD <1; P<0.05, Table 1).

Frequency domain analysis

Power spectral analysis reveals power components in the spectra at the frequency of oscillations in f_H and f_V. Low frequency components represent oscillations that occur more than every four heart beats or ventilations, and high frequency components occur less than every four intervals. When a low $\dot{M}_{O_2}$ was exhibited by the fish the power of high frequency components was fourfold the power of low frequency components (Fig. 3B, Table 2). When $\dot{M}_{O_2}$ was high due to stress, the power of low frequency components increased and the high frequency power was dramatically reduced. This resulted in a low:high power ratio of 1:1.4 (Table 2). In comparison, when a high $\dot{M}_{O_2}$ was induced by feeding, the high frequency power remained high and the low frequency power was elevated resulting in a ratio of 1:2.1. Vagotomy significantly reduced the total power of spectra for both f_H and f_V (Table 2), indicating that there were fewer oscillatory components in either f_H and f_V.

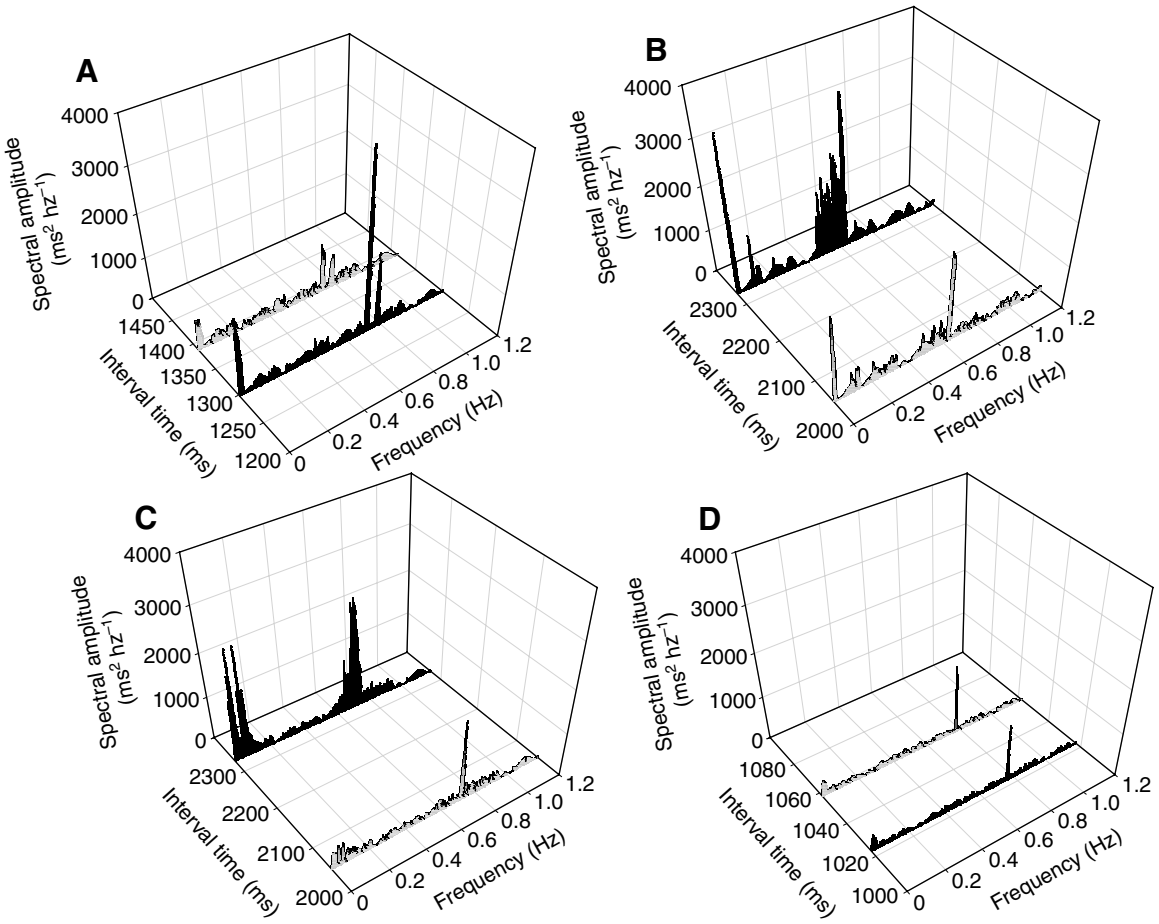

Fig. 3. Power spectra calculated using the discrete FT from raw ECG traces containing either 128 consecutive heart beats (black spectra) or ventilation cycles (grey spectra). All plots are from the same individual. (A) Spectra generated when $\dot{M}_{O_2}$ (1.8 mmol O_2 kg^{-1} h^{-1}), f_H (49 min^{-1}) and f_V (47 min^{-1}) were high due to surgery and placement in a respirometer; the peaks are very narrow, showing very little variability in either f_H or f_V. (B) After a period of no disturbance and starvation, $\dot{M}_{O_2}$ (0.91 mmol O_2 kg^{-1} h^{-1}), f_H (25 min^{-1}) and f_V (28 min^{-1}) were reduced dramatically and the f_H spectral peaks were spread within the bandwidth. (C) Ingestion of a 5%b.m. ration resulted in a very high $\dot{M}_{O_2}$ (2.11 mmol O_2 kg^{-1} h^{-1}), f_H (43 min^{-1}) and f_V (47 min^{-1}); the spectral peaks reduced in amplitude but remained with a broad bandwidth. (D) Bilateral vagotomy resulted in a moderately high $\dot{M}_{O_2}$ (1.41 mmol O_2 kg^{-1} h^{-1}), f_H (53 min^{-1}) and f_V (53 min^{-1}), and the spectral peaks were very narrow, indicating little variability.

Cross-spectral analysis was used to examine the relationship between in the oscillatory components in f_H and f_V under different levels of oxygen consumption (Fig. 4). A high $\dot{M}_{O_2}$ generated by either stress or feeding produced a high coherence (>0.69) in the 0–0.2 Hz frequency bin (Fig. 4A,C, Table 3). The phase relationship for this component was around 0°, showing that these low frequency oscillations occurred simultaneously in both f_V and f_H. During low $\dot{M}_{O_2}$ (Fig. 4B, Table 3) there was only coherence between f_V and f_H oscillations within the high frequency band (0.4–0.8 Hz). The phase relationship for this band was 30–50°. This showed that oscillations in f_H occurred with a short lag after a similar frequency oscillation in f_V (360° is one full ventilation cycle). When $\dot{M}_{O_2}$ was elevated due to feeding, the fish spectra still possessed this coherence in the high frequency band. The phase response was also similar. Sectioning the vagal nerve prevented any coherence or phase relationship between f_H and f_V, due to a lack of oscillatory components.

Discussion

The teleost *Paranotothenia angustata* showed distinctive oscillations in f_H caused by vagal inhibition of the heart beat, and consequently extension of the R-R interval. The oscillations in f_H closely followed changes in length of the ventilation cycle, and the rate association between these two components altered with metabolic demand. Abolition of these oscillations by sectioning the cardiac vagus resulted in a reduced rate of oxygen uptake compared with a similar f_H and f_V in an intact fish.

Changes in heart and ventilation rate with $\dot{M}_{O_2}$

The resting $\dot{M}_{O_2}$ and f_H values of *P. angustata* were comparable with ecologically similar fish at the same temperature (Jonhston and Battram, 1993; Campbell et al., 2004). $\dot{M}_{O_2}$ increased in response to both stress and digestion and the metabolic rise was accompanied by increased f_H and gill ventilation, presumably to increase oxygen delivery to the tissue. When $\dot{M}_{O_2}$ was elevated, f_H was greater than f_V, with the

Table 2. *Total and relative power of the spectral components calculated using power spectral analysis from 128 consecutive heart beats or ventilation cycles from fish with different rates of oxygen consumption*

	Power ($\times 10^3$ ms^2)		
Group	Total	Low frequency	High frequency
f_H			
A	17.6±6.5	3.5±1.60	9.1±0.98
B	28.3±4.2*	5.2±1.50	23.1±3.26*
C	31.2±8.9*	9.1±1.30	19.1±3.03*
D	0.2±1.5	1.1±0.89	1.1±0.75
f_V			
A	4.2±1.60	1.1±0.20	3.1±0.09
B	8.2±1.85*	2.1±0.15*	6.1±0.11*
C	7.7±1.45*	1.9±0.15*	5.8±0.11*
D	2.8±1.20	0.4±0.11	1.4±0.07

f_H, heart-beats; f_V, ventilation cycles (means ± s.e.m., N=8) from fish with (A) a high $\dot{M}_{O_2}$ due to surgery and placement in a respirometer; (B) a low $\dot{M}_{O_2}$ after 120 h undisturbed starvation; (C) a high $\dot{M}_{O_2}$ due to ingestion of a 5%b.m. ration; (D) a high $\dot{M}_{O_2}$ due to bilateral vagotomy.

Each trace was divided exactly in half between zero and the Nyquist limit, and designated low or high frequency status. Multivariate ANOVA assumes that the mean data for each physiological state is significantly different, and homogeneous groups are indicated by asterisks.

reverse occurring at a low $\dot{M}_{O_2}$. This is an important observation as it implies that a f_H:f_V 1:1 synchrony was only exhibited by the fish during intermediate levels of oxygen demand. Log transformation of $\dot{M}_{O_2}$ and fitting of linear regression confirmed that f_H and f_V would be equal at an oxygen consumption of ~1.3 mmol O_2 kg^{-1} h^{-1}. The maximum $\dot{M}_{O_2}$ shown during the study was 1.99±0.02 mmol O_2 kg^{-1} h^{-1} for fed fish and 1.82±0.03 mmol O_2 kg^{-1} h^{-1} for starved fish. Consequently, as ventilation and heart rate are coupled at an $\dot{M}_{O_2}$ that is intermediate between stressed and undisturbed fish, CRC cannot be essential for optimising gas exchange, as previously proposed, when f_H and f_V would be matched at maximum $\dot{M}_{O_2}$. Therefore, 1:1 synchrony to match the blood and water pulse at the gill surface was not the preferred strategy used by *P. angustata* to optimise oxygen delivery. Instead, the blood pulse through the gills was at a higher rate than periodic flushing of water over the gills. This may have been due to anatomical or physiological constraints, and agrees in part with the mathematical model of blood and water pulsatility proposed by Malte (Malte, 1992). The $\dot{M}_{O_2}$ at which coupling occurs may reflect routine metabolism under natural conditions, where fish are unlikely to be completely undisturbed and $\dot{M}_{O_2}$ accommodates small levels of activity and digestion. Indeed, the minimum f_H recorded by dataloggers from *P. angustata* released into the wild was 34 min^{-1} (Campbell et al., 2005b) compared to 25 min^{-1} in rested fish in the present study, by extrapolation a difference in $\dot{M}_{O_2}$ of ~0.5 mmol O_2 kg^{-1} h^{-1}.

Interestingly, after feeding the fish exhibited a higher $\dot{M}_{O_2}$ at a lower f_H and f_V than was observed in starved fish when $\dot{M}_{O_2}$ was elevated due to handling stress. Moreover, bilateral vagotomy further reduced the level of $\dot{M}_{O_2}$ for a given f_H and f_V. Therefore, rate changes in the heart beat and the ventilation cycle cannot alone account for altered oxygen uptake by the fish, which may also be modulated, e.g. by stroke volume or extraction efficiency.

Changes in heart beat and ventilation pattern with $\dot{M}_{O_2}$

After placement in the respirometry chamber, the scattergram plots of heart beat intervals and ventilation cycles showed a narrowing in the spread of these data. In mammalian studies the shape of the scattergram has been characterised into functional classes that indicate physiological state (Kamen et al., 1996) by revealing the differing temporal bases due to changes in parasympathetic (short-term) and sympathetic (long-term) modulation (Woo et al., 1992; Tulppo et al., 1996). Using this model we interpret the scattergrams to show that after placement in the respirometery chambers there is a large increase in sympathetic, and reduction in parasympathetic, activity. Acclimation to the respirometry chamber greatly increased f_H beat-to-beat variability, and scattergram interpretation suggests an increase in parasympathetic tone. Feeding reduced the spread in the scattergram of f_H data points. The shape of the plot reveals a very different variability from that observed in a fish under conditions of experimental stress, and suggests a difference in the action of

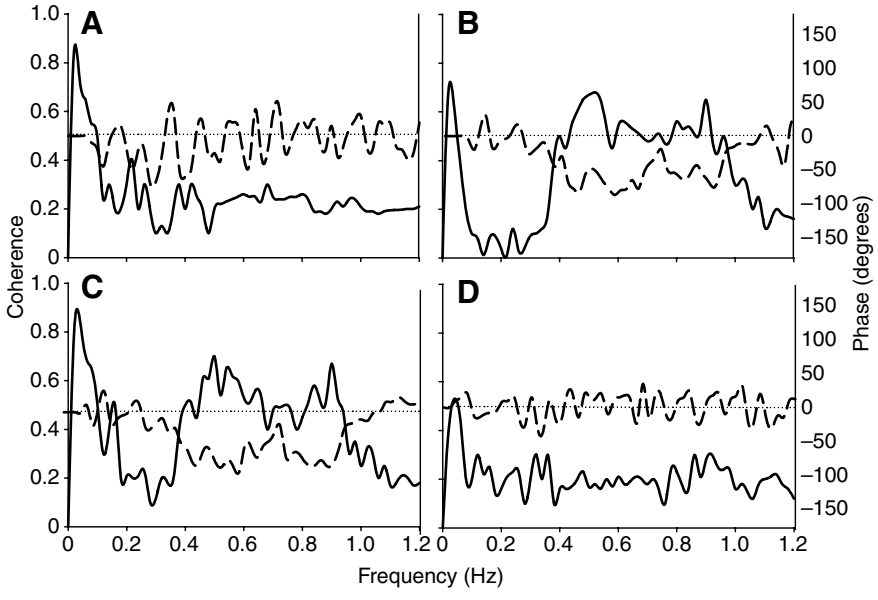

Fig. 4. Spectra from cross-spectral analysis, describing the coherence (solid lines) and phase relationship (broken lines) of frequency oscillations between f_H and f_V. All plots are from a single individual with: (A) a high $\dot{M}_{O_2}$ due to surgery and placement in a respirometer; (B) a low $\dot{M}_{O_2}$ after 120 h undisturbed starvation; (C) a high $\dot{M}_{O_2}$ due to ingestion of a 5%b.m. ration; (D) a high $\dot{M}_{O_2}$ due to bilateral vagotomy.

Table 3. *The coherence and phase relationship between power spectra calculated from f_H and f_V recorded from fish with different rates of oxygen consumption*

	Coherence			Phase		
Frequency bin	0–0.2	0.2–0.4	0.4–0.8	0–0.2	0.2–0.4	0.4–0.8
A	0.78±0.10*	0.22±0.04	0.28±0.02	–12±22	NA	NA
B	0.24±0.04	0.04±0.08	0.54±0.10*	NA	NA	–58±5*
C	0.69±0.04*	0.31±0.06	0.58±0.07*	0±12	NA	–57±9*
D	0.35±0.05	0.21±0.03	0.13±0.04	NA	NA	NA

f_H, heart-beats; f_V, ventilation cycles (means ± s.e.m., N=8) from fish with (A) a high $\dot{M}_{O_2}$ due to surgery and placement in a respirometer; (B) a low $\dot{M}_{O_2}$ after 120 h undisturbed starvation; (C) a high $\dot{M}_{O_2}$ due to ingestion of a 5%b.m. ration; (D) a high $\dot{M}_{O_2}$ due to bilateral vagotomy. Each spectrum was divided into low, mid and high frequency bins to describe the f_H and f_V oscillation association.

NA, data where the coherence was not high enough (i.e. <0.4) to consider a valid phase response. Multivariate ANOVA assumes that the mean data for each physiological state is significantly different, and homogeneous groups are indicated by asterisks.

sympathetic and parasympathetic tonus on the heart. The variability patterns of both f_H and f_V dramatically changed under the different induced levels of metabolism, and therefore the association between the heart beat and ventilation cycle would also have been altered. This would have changed the counter current association between the lamellae blood pulse and water flow across the gill. Sectioning the vagal nerve abolished all variability in f_H, and the heart beat and ventilation cycle association would have been very different from the intact animal.

Cardio-respiratory coupling

PSA applied to mammalian HRV signals shows a fundamental component in the spectra at the frequency of ventilation (Saul, 1990). The same technique applied to fish has not uncovered a similar component (Armstrong et al., 1989; De Vera and Priede, 1991; Altimiras et al., 1995; Campbell et al., 2004). This is a result of the close rate association between f_H and f_V in fish, and therefore it is not possible for f_V to modulate f_H in an analogous manner to RSA that is evident in mammals (Campbell et al., 2005a). By using the modified PSA technique of Campbell et al. (Campbell et al., 2006), however, it is evident that in *P. angustata* there are common oscillatory components in the spectra when f_H and f_V are recorded simultaneously. These components also fluctuate in frequency and amplitude depending on the metabolic demand of the fish. At a low $\dot{M}_{O_2}$ (resting fish) a large power of high frequency components was present in the f_H spectrum, and similar components were also shared by the f_V spectrum. Elevation of $\dot{M}_{O_2}$ by the stress response, but not by feeding, abolished these components. If the high frequency components in the f_H spectrum are a direct consequence of vagal influences on the sinoatrial node, as suggested for mammals (Medigue et al., 2001), then whilst vagal activity is withdrawn during periods of stress, it remains high after feeding. An elevated heart rate with a high vagal tone has also been observed in the Boa constrictor snake after feeding, suggesting an additional factor modulating cardiac activity may be released from the gut in lower vertebrates (Wang et al., 2001).

The coherence and phase relationship of f_H and f_V components showed low frequency oscillations that occurred virtually simultaneously. These were probably caused by physiological feedback control other than CRC, such as humoral or vasomotor responses. The high frequency components in the f_H spectra, observed in fish with a low $\dot{M}_{O_2}$ and after feeding, had a strong coherence but were 30° to 50° out of phase. If one complete ventilation cycle is 360°, the f_H oscillation (caused by vagal activity delaying firing of the cardiac pacemaker) followed variability in the ventilation cycle by approximately one seventh of a cycle. Intriguingly, this cardio-respiratory coupling occurred both when f_H was greater than f_V (high $\dot{M}_{O_2}$ after feeding) and when f_H was less than f_V (low $\dot{M}_{O_2}$), and demonstrates its importance over rate changes that may be determined by other factors (e.g. sympathetic drive).

Vagal control

The vagus appeared to have a predominant controlling effect on both heart rate and heart rate variability. Abolition of this variability by bilateral cardiac vagotomy confirms its action, and similar responses have been seen in many other vertebrates (Taylor et al., 1999). After vagotomy the fish did not attain as high an $\dot{M}_{O_2}$ for a given heart rate as when the vagus was intact; however, overall f_H, f_V and $\dot{M}_{O_2}$ remained at higher rates than observed in intact resting fish. This suggests a role for the cardiac vagus in influencing fish metabolism that may be directly or indirectly mediated through cardiac output. Bilateral vagotomy has also been shown to elevate f_H and $\dot{M}_{O_2}$ in the pigeon (Hissa et al., 1995), which displays features considered indicative of increased cellular activity. Nevertheless, the reasoning for increased metabolic requirements in a vagotomised animal remains poorly understood.

Interestingly, sectioning of the vagus not only abolished variability in f_H but also in f_V, with the exception of a few scattered points that may indicate sporadic coughing. Vagotomy increased breathing amplitude in the neotropical fish tambaqui (Milsom et al., 2002), but the reduction in variability of f_V by cardiac vagotomy is a novel observation. It indicates a feedback influence on the ventilatory drive from the heart, possibly involving a branchial equivalent of the pulmonary stretch receptor reflex of mammals, and merits further investigation.

Conclusions

Heart rate variability patterns in *P. angustata* included cardio-respiratory coupling that was abolished by bilateral vagotomy, after which $\dot{M}_{O_2}$ was significantly reduced for a given f_H, suggesting a role for the cardiac vagus in influencing metabolism. Although our data cannot demonstrate that CRC

directly mediated oxygen uptake across the gill, they show for the first time in a teleost that centrally coordinated blood flow in the lamellae and gill irrigation varies with both physiological state and metabolic demand.

List of abbreviations

%b.m.	% of body mass
CRC	cardio-respiratory coupling
ECG	electrocardiogram
f_H	heart rate
FT	Fourier transform
f_V	ventilation rate
HF	higher frequency half of the spectrum
HRV	heart rate variability
LF	lower frequency half of the spectrum
lt	long-term
$\dot{M}_{O_2}$	rate of oxygen consumption
OA	axis from the origin
PSA	power spectral analysis
R-R	time interval between heart beats
RSA	respiratory sinus arrhythmia
SDRR	standard deviation of the R-R interval
SDSD	standard deviation of the successive differences of the R-R (or V-V) intervals
SDVV	standard deviation of the V-V interval
st	short-term
VRV	ventilation rate variablility
V-V	time interval between intervalopercular movements

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